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<p>(54) Title: IMMUNOSUPPRESSION</p> <p>(57) Abstract</p> <p>The present invention relates, in general, to immunosuppression and, in particular, to a method of inducing an immunosuppressive effect. The invention further relates to a method of screening compounds for immunosuppressive activity.</p>			

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**IMMUNOSUPPRESSION**

This application claims priority from Prov. Appln. No. 60/038,558, filed March 3, 1997, which is  
5 incorporated in its entirety by reference.

**TECHNICAL FIELD**

The present invention relates, in general, to  
10 immunosuppression and, in particular, to a method of inducing an immunosuppressive effect. The invention further relates to a method of screening compounds for immunosuppressive activity.

**BACKGROUND**

$\text{Ca}^{2+}$ /calmodulin-dependent protein kinase IV (CaMKIV) is a monomeric multifunctional enzyme that is expressed only in subanatomical portions of the brain, 20 T lymphocytes and postmeiotic male germ cells. It is present in the nucleus of cells in which it is expressed (Jensen et al, Proc. Natl. Acad. Sci. USA 88:2850 (1991)). CaMKIV phosphorylates and activates the cyclic AMP response element binding proteins CREB and CREM $\tau$  in 25 a manner analogous to protein kinase A (Matthews et al, Mol. Cell. Biol. 14:6107 (1994); Sun et al, Genes Dev. 8:2527 (1994); Enslen et al, J. Biol. Chem. 269:15320 (1994)).

In the absence of  $\text{Ca}^{2+}$ /calmodulin, CaMKIV is 30 inactive. Activation requires three events: i) binding of  $\text{Ca}^{2+}$ /calmodulin; ii) phosphorylation of a single

threonine residue present in the activation loop by a separate protein kinase that is also  $\text{Ca}^{2+}$ /calmodulin-dependent; and iii) autophosphorylation of serine residues present in the extreme N-terminus that is 5 required to relieve a novel form of autoinhibition.

The gene for rat CaMKIV has been cloned and found to span 42 kb of DNA. The gene encodes 3 proteins namely, the  $\alpha$  and  $\beta$  forms of CaMKIV that differ only in that the  $\beta$  form contains a 28 amino acid N-terminal 10 extension as well as calspermin (Sun et al, J. Biol. Chem. 270:29507 (1995)). Calspermin is the C-terminal 169 amino acids of CaMKIV that binds  $\text{Ca}^{2+}$ /calmodulin and is expressed only in postmeiotic male germ cells. The promoter for calspermin resides in the penultimate 15 intron of the CaMKIV gene and is regulated by two CREs. Available data suggest that rearrangement of chromatin during meiosis together with the expression of CREM $\tau$  at high levels are sufficient to control expression of the calspermin promoter during spermatogenesis. On the 20 other hand, the developmental expression of CaMKIV in cells of the brain and T cells appears to be controlled by thyroid hormone mediated via the thyroid hormone receptor  $\alpha$ .

Prior to the present invention, the specific role 25 of CaMKIV in T cell activation had not been defined and the potential importance of CaMKIV as a target for immunosuppressive agents had not been suggested. Indeed, currently available immunosuppressive drugs, as well as new generation drugs presently under 30 development, target calcineurin. Since calcineurin is present in all cells, not just T cells, these drugs are

associated with a plethora of side effects. The present invention provides a method of identifying immunosuppressive agents that target CaMKIV and that are T cell specific and thus substantially free of adverse 5 side effects.

OBJECTS AND SUMMARY OF THE INVENTION

10 It is one object of the invention to provide a method of inducing an immunosuppressive effect, while avoiding undesirable side effects.

15 It is another object of the invention to provide a method of screening compounds for immunosuppressive activity.

20 It is a further object of the invention to provide a method of inhibiting CREB phosphorylation specifically in T cells.

25 The foregoing objects are met by the present invention which results, at least in part, from the recognition that inhibition of CaMKIV prevents phosphorylation of CREB and thereby activation of T cells.

Further objects and advantages of the present invention will be clear from the description that 25 follows.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1C. Fig. 1A. Diagrammatic representation of 30 the two transgenes used to generate transgenic mice. The left panel depicts the hCaMKIV construct and the amino

acid substitution used to generate the kinase inactive form. The right panel shows the calspermin construct. Fig. 1B. Southern blot analysis of transgenic mice. The left panel depicts two founding lines of hCaMKIVkin(-) mice while the right panel shows one of three founding lines of the calspermin overexpressing mice. Fig. 1C. Expression of endogenous CaMKIV, hCaMKIVkin(-), and calspermin. The left panel shows the expression of CaMKIV in a control mouse (center lane) and from the two different founding lines (left lane is line K; right lane is line I). The center panel shows the expression of calspermin in one of the founding lines of mice [note that the calspermin antibody also recognizes CaMKIV]. The right panel shows expression of the hCaMKIVkin(-) in thymic T cells from line I transgenic mice utilizing an antibody that only recognizes the human form of CaMKIV. Expression of the hCaMKIV is absent in transgenic splenocytes as well as in control thymic and splenic cells.

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Figures 2A-2B. Fig. 2A. Cellularity of thymi and survival of thymic T cells from transgenic mice. Cells were harvested, counted and plated for survival assays. The left histogram depicts the cellularity of the thymus from both transgenic lines and a control line of mice (n=14; P<0.01). The right histogram shows the rate of survival in culture of these same cells (n=34; P<0.01). Fig. 2B. Cellularity and survival of splenocytes that were isolated and treated identically as were the thymic T cells.

Figure 3. Distribution of CD4 and CD8 T cells. Cells were isolated from thymi and spleens of transgenic and control mice and stained for flow cytometry using PE conjugated anti-CD4 and FITC conjugated anti-CD8 antibodies. The upper panel shows the distribution of cells from the thymus while the lower panel shows the distribution of cells from the spleen. This Figure is representative of the results obtained from 6 independent experiments.

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Figures 4A and 4B. CREB phosphorylation is inhibited in T cells that express mutant hCaMKIV. Fig. 4A. Thymocytes and splenocytes from control and lck-CaMKIV<sup>kin(-)</sup> transgenic mice were either unstimulated (uns) or stimulated with PMA + ionomycin or with a-CD3 for 1, 5, and 30 min. Cell lysates were then subjected to western blot analysis using antibody specific for CREB phosphorylated on Ser-133 (a-pCREB) or antibody recognizing both phosphorylated and unphosphorylated CREB (a-CREB). The arrow indicates the position of CREB. The faster migrating inducible band is tentatively identified as ATF-1. Shown is one representative experiment. (n=9). Fig. 4B. Thymocytes and splenocytes from lck-calspermin transgenic mice were either unstimulated or stimulated with PMA + ionomycin for 5 and 30 min. Levels of phosphorylated CREB were then determined as described in Fig. 4A. Shown is one representative experiment. (n=2).  
30 Figure 5. Fos B induction is inhibited in T cells that express mutant hCaMKIV. Thymocytes from control and

lck-CaMKIVkin(-) transgenic mice were stimulated with PMA + ionomycin for the indicated time. Total RNA was then extracted and subjected to northern blot analysis using a Fos B-specific probe. To assess RNA sample loading the blot was striped and reanalyzed with a probe for glucose-3-phosphate dehydrogenase (GAPDH). Shown is one representative experiment. (n=2).

Figures 6A and 6B. Induction of IL-2 and IL-2Ra are inhibited in T cells that express mutant hCaMKIV.

Fig. 6A. IL-2 induction. IL-2 secretion from control and lck-CaMKIVkin(-) and lck-calspermin transgenic thymocytes was quantified after stimulation for 48 h with PMA + ionomycin. Values represent the average +/- SD of one representative experiment performed in triplicate (n=5). Fig. 6B. IL-2Ra (CD25) induction. Thymocytes from control and lck-CaMKIVkin(-) and lck-calspermin transgenic mice were stained for CD25, and analyzed by FACS on a flow cytometer before (unshaded curve) and after stimulation for 30 h with PMA + ionomycin (shaded curve). Shown is one representative experiment. (n=4).

Fig. 7 Construct prepared for use in mouse CaMKIV knockout gene studies.

#### DETAILED DESCRIPTION OF THE INVENTION

CaMKIV is expressed exclusively in subanatomical portions of the brain (particularly enriched in mature granule cells), meiotic (but not mitotic) male germ

cells and lymphocytes of thymic origin (ie T cells). A primary target for CaMKIV in mature T cells is the transcription factor CREB. Phosphorylation of CREB by CaMKIV (at Ser-133) results in transcriptional 5 activation of the intermediate early genes (members of the fos and jun families) which are responsible for activation of genes encoding cytokines, such as interleukin 2 (IL-2). The production of IL-2 is required for activation of T cells by serving as a 10 requisite mitogen. The present invention is based on the realization that inhibition of CaMKIV-dependent phosphorylation of CREB in T cells results in immunosuppression. This realization and the fact that the only cells other than T cells that express CaMKIV 15 are protected by a blood brain barrier (ie testis and brain), make possible the identification of T-cell-specific immunosuppressants.

The present invention relates to a method of screening compounds for immunosuppressive activity. In 20 one embodiment, the method comprises:

- 25 i) contacting CaMKIV and a substrate therefor, in the presence and absence of a test compound, under conditions such that CaMKIV-dependent phosphorylation of the substrate can be effected, and
- ii) determining, directly or indirectly, the level 25 of phosphorylation of the substrate, wherein a reduction in phosphorylation of the substrate in the presence of the test compound is indicative of an immunosuppressive agent.
- 30 The method of the invention can be carried out in a cell free system or using cells in culture.

Advantageously, a test compound is screened for immunosuppressive activity first in a cell free system and subsequently in an intact cell system. Cell free systems can include, in addition to CaMKIV and substrate, CaMKIV kinase, calmodulin and calcium, as well as a phosphate donor. The concentrations of the various system components can vary, however, one skilled in the art can readily optimize reaction conditions based on the guidance provided in the Examples that follow. The reaction can be carried out in a standard kinase buffer, such as those described in the Examples that follow and in the citations incorporated herein by reference.

The substrate used in the cell free system is advantageously a peptide substrate. While CREB can be used, alternative substrates, such as syntide-2 from Bachem, can also be employed. Phosphate donors suitable for use in the present invention include ATP, a radiolabeled form, such as [ $\gamma$ -<sup>32</sup>P]ATP, being preferred.

The effect of the test compound on the CaMKIV-dependent phosphorylation can be determined using a variety of approaches well known in the art. When <sup>32</sup>P-ATP is used as the phosphate donor, the extent of phosphorylation of the substrate can be determined by monitoring the amount of <sup>32</sup>P associated with the substrate.

In a specific embodiment of the above-described method, a test compound is tested for its ability to competitively inhibit CaMKIV phosphorylation of a peptide substrate (e.g., GS10); standard protein kinase assays can be used (see generally Selbert et al, J.

Biol. Chem. 270:17616 (1995)). For example, a reaction mixture comprising the test compound, the peptide substrate, CaMKIV, calcium, calmodulin, CaMKIV kinase and phosphate donor (e.g., bearing a detectable label, 5 such as a radioactive label) is prepared and incubated under conditions such that the kinases present in the mixture can effect phosphorylation of their respective substrates (a constitutively activated form of CaMKIV can be used in the reaction mixture in lieu of CaMKIV, 10 in which case the CaMKIV-activating components (e.g., CaMKIV kinase) need not be present). The reaction mixture is then processed so as to eliminate labeled phosphate not associated with substrate (i.e., non-incorporated labeled phosphate). For example, the 15 reaction mixture can be applied to an ion exchanger, for example, an ion exchange filter (e.g., a P81 filter). The ion exchanger (e.g., filter) is then washed and the amount of detectable label associated therewith determined and compared with the amount of detectable 20 label associated with a control ion exchanger (i.e., an ion exchanger to which a reaction mixture as described above but minus test compound, is applied). A reduction in the amount of detectable label associated with the ion exchanger (and thus with substrate) in the presence 25 of the test compound (relative to the control) is indicative of an immunosuppressive agent.

CaMKIV must bind  $\text{Ca}^{2+}$ /calmodulin to be active. Further, CaMKIV must be phosphorylated on Thr-200 (in the human form of the enzyme) by CaMKIV kinase. This 30 phosphorylation can be readily detected, for example, by separating the components of the reaction mixture (as

described above), for example, on a polyacrylamide gel, and quantifying the amount of detectable label incorporated into CaMKIV, for example, by phosphoimaging. Since phosphorylation of CaMKIV is required for CaMKIV activation, a test compound that inhibits CaMKIV kinase can also be expected to be an effective immunosuppressive agent. The effect of a test compound on the phosphorylation events (e.g., phosphorylation of CaMKIV by CaMKIV kinase or phosphorylation of CaMKIV substrate by CaMKIV) can be measured in a single reaction. To effect this simultaneous screen,  $\text{Ca}^{2+}$ /calmodulin, CaMKIV kinase, CaMKIV, substrate for CaMKIV and labeled phosphate donor (e.g.,  $^{32}\text{P}$  ATP) can be combined with a standard protein kinase assay reaction mixture. After incubation, an aliquot of the resulting reaction mixture is applied, for example, to a P81 filter and processed as described above for determination of CaMKIV substrate phosphorylation. This provides an assessment of CaMKIV activity. A further aliquot is, for example, run on a polyacrylamide gel and the amount of labeled phosphate incorporated into the CaMKIV quantified, for example, by phosphoimaging. This provides an assessment of CaMKIV kinase activity. CaMKIV is the only known substrate of the CaMKIV kinase, except for CaMKI. At present, CaMKI does not have a known physiological function. (See generally assay details provided in Selbert et al, J. Biol. Chem. 270:17616 (1995)).

As indicated above, test compounds screened in the cell free system can also be screened using a system based on cells in culture. Cells that contain CREB and

CaMKIV naturally (eg T cells) can be used in such screens, as can cells resulting from engineering. Mammalian as well as non-mammalian engineered cells can be used, lymphocytes or fibroblasts transfected with a 5 CaMKIV encoding sequence being examples, as are yeast cells transfected with sequences encoding the required proteins. (See Sun et al, Genes & Develop. 8:2527 (1994).) Cells can be stimulated using a variety of approaches, including use of a calcium ionophore or 10 occupancy of the T cell receptor. As in the case of the cell free system, the immunosuppressive activity of a test compound can be determined by assessing the extent to which the substrate (e.g., CREB) is phosphorylated. While a variety of techniques can be used (including 15 quantitating the transfer of  $^{32}\text{P}$  from a radiolabeled phosphate donor to the substrate), the use of antibodies specific for the phosphorylated form of the substrate (e.g., antibodies specific for the phosphorylated form of CREB) is preferred (see 33). 20 Compounds demonstrated to have immunosuppressive activity based on one or more of the above-described screens can be modified as appropriate to minimize toxic effects and the like. Since T cell specificity is desired, immunosuppressive compounds can also be 25 derivatized, if necessary, to ensure that they do not cross the blood brain/testis barrier. 30 Screening procedures such as those described above are useful for identifying agents for their potential use in pharmacological intervention strategies including those in which FK506 and cyclosporin A are typically employed. Examples of areas in which such agents will

find applicability include treatment of autoimmune diseases, including multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosis, and treatment of patients undergoing organ and tissue 5 (including bone marrow) transplantation.

The present invention also relates to pharmaceutical compositions comprising, as active agent, compounds selected using one or more of the above-described screening protocols. Such compositions 10 include the active agent(s) in combination with a pharmaceutically acceptable carrier. The amount of active agent in the composition can vary with the agent, the patient and the effect sought. Likewise, the dosing regimen, including mode of administration, will vary 15 depending on the composition and the disease/disorder to be treated.

Certain aspects of the present invention are described in greater detail in the non-limiting Examples that follow. One skilled in the art will appreciate 20 from a reading of the foregoing and the Examples that the transgenic animals described herein can be used to screen compounds for immunostimulatory potential.

#### EXAMPLES

25

The following experimental details relate to specific Examples that follow (numbers in parentheses refer to References below).

## Materials:

Phorbol-12 myristate-13 acetate (PMA) and ionomycin were obtained from Calbiochem (La Jolla, CA). [<sup>125</sup>I] protein-A, [ $\alpha$ -32P]deoxy-CTP and ECL chemiluminescent reagents were purchased from Amersham (Arlington Heights, IL). Phycoerythrocin and FITC conjugated antibodies for the murine CD4, CD8, and CD25 complexes as well as all tissue culture reagents came from Gibco (Grand Isle, NY). Anti-calspermin antibodies were produced in rabbits housed on site at the Duke University vivarium and had characteristics similar to the antibodies described by Ono et al. (29). A bacterially expressed form of the rat calspermin protein was utilized as the antigen. Two independently derived anti-CaMKIV polyclonal antibodies were also obtained. One was a gift from Talal Chatila (17) the other generated commercially by Immuno-Dynamics Incorporated (La Jolla, CA). Both antibodies were produced in rabbits against identical synthetic peptides that consisted of the C-terminal 17 amino acids of the human form of CaMKIV. Anti-CREB and anti-phosphoCREB antibodies were obtained from Upstate Biotechnology Incorporated (Lake Placid, NY). ELISA reagents for the measurement of murine interleukin-2 (IL-2) production as well as the anti- $\alpha$ CD3 monoclonal antibody (145.2C11) were provided by Pharmingen (San Diego, CA). All other reagents were of molecular biology grade unless otherwise specified

Transgene Construction and Generation of Mice:

Targeted expression of the transgenes to murine thymocytes was accomplished by utilizing the shuttle vector p1017 [a gift from R. Perlmutter, University of Washington, Seattle] (25). This vector contains the sequence for the mouse proximal lck promoter plus additional sequence derived from the human growth hormone gene (hGH) and has been shown to initiate high level expression of cDNAs specifically in thymic T cells of transgenic mice (27). A 1.5 Kb cDNA for the human form of Ca<sup>2+</sup>/calmodulin dependent protein kinase type IV (hCaMKIV) that had been mutated to result in a catalytically inactive protein (23) and a 0.5 Kb cDNA for the rat form of calspermin (29) were isolated from plasmids by the appropriate restriction digests and subcloned separately into a BamHI site between the lck promoter and the hGH sequences of the p1017 vector as represented diagrammatically in Figure 1A. After confirming that the cDNAs were in the correct orientation by DNA sequencing, the transgenes were linearized and isolated by restriction digestion with NotI (Boehringer Mannheim, Indiana, IN) followed by electrophoresis in a 1% agarose gel (FMC Corp., Rockland, MD). Both transgenes were purified from the agarose using Prep-a-Gene (Bio-Rad, Richmond, CA), aliquoted in 10 mM Tris and 0.1 mM EDTA buffer and stored at -20C for subsequent microinjection.

B6SLF1/J mice (Jackson Laboratories, Bar Harbor, ME) were used to generate the transgenic mice, because they produce excellent yields of viable eggs for microinjection [Cheryl Bock, Director of the Duke

Transgenic Core Facility, personal communication]. Ovulation of female mice, male stud service, collection of eggs, microinjection of DNA and reimplantation of fertilized eggs was done following standard methods 5 previously described (50) and was performed by the Duke Comprehensive Cancer Center Transgenic Core Facility. Mice that expressed the correct DNA fragment as determined by Southern blot analysis were then bred to nontransgenic B6SLF1/J mice in order to establish viable 10 lines for analysis. All mice were housed at the Duke Cancer Center Isolation Facility under a 12 hour light-dark cycle. Food and water were provided ad libidum and all care given to the mice was in compliance with the NIH guidelines for the care and use of laboratory 15 animals.

DNA and RNA Analysis:

Genotyping of founder mice and subsequent generations of mice was done as previously described by 20 Southern blot analysis (51). In this procedure, 10ug of genomic DNA was digested with SpeI (Boehringer Mannheim) for 12h at 37C electrophoresed through a 1% agarose gel and blotted onto Zeta-Probe membranes (Bio-Rad, Richmond, CA). 32P randomly labeled probes were used to 25 confirm the presence of the transgene and to ascertain copy number. For the lck-hCaMKIVkin(-) mice a 500bp BamHI-BglII fragment derived from the hCaMKIV cDNA 3+ end was utilized as the probe. In the case of the lck-calspermin transgene we utilized the entire 522bp cDNA 30 encoding rat calspermin. It should be noted that the SpeI restriction site used for Southern analysis was

engineered into the p1017 plasmid at both the extreme 5+ and 3+ ends (25, 27) and does not exist in the wild type locus for either CaMKIV or calspermin. Hence, while the cDNA probes also hybridized to the wild type locus only 5 a single band was detected in Southern blots because the wild type band was substantially larger than the transgene. Total RNA was isolated from tissue after extraction with Ultraspect<sup>TM</sup> total RNA isolation reagent (Biotex Laboratories, Houston, TX). RNA was subjected 10 to electrophoresis through 1.5% agarose-formaldehyde gels (5 $\mu$ g/lane) and blotted onto Zeta-Probe membranes (Bio-Rad, Richmond, CA.). RNA blots were then hybridized to a 32P randomly labeled cDNA fragment corresponding to the appropriate mRNA. The Fos B probe 15 was made from a 1.6 kb Hind3 fragment of murine Fos B. The GAPDH probe was from a 780 bp Pst1/Xba1 fragment of human GAPDH. Hybridization was carried out in 500 mM sodium phosphate (pH 7.2), 7% sodium dodecyl sulfate (SDS), and 1 mM EDTA at 65°C for 18h. Washes were done 20 at 65°C in 40 mM sodium phosphate and 1% SDS until background level radioactivity was undetectable. Visualization of blots was accomplished by exposure of the blot to Kodak XAR film (Rochester, NY) at -70°C for up to 24h.

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Primary cell culture:

Primary cultures of thymic or splenic T cells were established from mice between the ages of 21 and 30 days of age for all experiments. Mice were killed by 30 cervical dislocation and the thymus or the spleen was harvested. Cells were teased from the organs by placing

the organs between two pieces of 40xx nytex nylon mesh (Tetko, Kansas City, MO) submerged in RPMI-1640 (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 0.5% penicillin, 0.5% streptomycin, 1x non-  
5 essential amino acids and 0.001M 2-mercaptoethanol at 4C and gently pushing down on top of the mesh to rupture the organs. The cells were collected from the supernatant fluid and the remaining tissue was removed and discarded. This procedure resulted in a T-cell  
10 purity of >95% from the thymus as assessed by flow cytometry. Splenic T cells were not fractionated on columns but were differentiated from B cells based upon the size differential when counting by trypan blue exclusion. This method yielded consistent results and  
15 flow cytometry was employed to confirm the presence of large numbers of T cells. After two successive washes in RPMI-1640 media the cells were counted on a hemocytometer by trypan blue exclusion to determine both the number of cells as well as the fraction representing  
20 live cells before aliquoting them for experiments.

Survival assays were carried out on aliquots of cells from spleen or thymus. Cells were collected by centrifugation at 1000 rpm for 5 min at 4C, and resuspended in Dullboco's modified Eagle's medium (GIBCO, Grand Island, NY) supplemented with 10% FBS and 0.5% penicillin, 0.5% streptomycin. Cells were then recounted on a hemocytometer by trypan blue exclusion, and 4x10<sup>6</sup> live cells were plated in pentuplicate in 24 well Falcon brand plates (Becton Dickenson, San Jose, CA) containing 2ml of DMEM. The cells were then incubated at 37C in 95% O<sub>2</sub>/5% CO<sub>2</sub>. After 30h the cells

were harvested and counted on a hemocytometer by trypan blue exclusion to determine the rate of survival.

FACS Analysis:

5 All FACS analyses were carried out at the Duke Comprehensive Cancer Center Flow Cytometry Core Facility. Experiments were analyzed within 24h after completion using a Becton-Dickinson Model FACScan with data analysis done using the Cell Quest program (Becton-  
10 Dickinson, San Jose, CA).

CD4/CD8 determinations for thymic and splenic T cells were carried out as previously described (52). Briefly, 1x10<sup>6</sup> T cells were placed into 10 X 75 mm tubes (Becton Dickinson, San Jose, CA) on ice. The cells were  
15 washed 2 times with phosphate buffered saline containing 5% FBS (FACS buffer). The supernatant fluid was aspirated and 100 $\mu$ l of FACS buffer was added containing either PE conjugated anti-CD4 (diluted 1:50) or FITC conjugated anti-CD8 (diluted 1:50) or both PE-CD4 and  
20 FITC-CD8. The reaction was allowed to proceed 20 min at 4C before the reaction was terminated by the addition of fresh FACS buffer. Cells were then briefly washed with fresh FACS buffer and pelleted at 1000 rpm 2 times. The cells were then fixed with PBS containing 1%  
25 formaldehyde.

Quantification of the murine interleukin-2 (IL-2) receptor on T cells was accomplished by taking an aliquot of 4x10<sup>6</sup> live cells, dividing them in half, and plating the paired samples containing 2x10<sup>6</sup> live cells  
30 each into 24 well Falcon Brand plates (Becton-Dickinson) containing fully supplemented RPMI-1640. The final

volume of each well was then adjusted to 2 ml. One half of each paired cell sample was left unstimulated while the other half was stimulated with PMA (10ng/ml) and ionomycin (0.5ug/ml). After 30h the cells were 5 harvested, washed in FACS buffer as described above, and immunostained for FACS analysis using FITC conjugated anti-CD25 antibody (Gibco, Grand Island, NY.) which recognizes the IL-2 receptor  $\alpha$ -chain .

10 Protein and Cytokine Analysis:

Detection of proteins on western blot was accomplished by either standard radioactivity methods or by chemiluminescence. Expression of protein from the transgene was examined by first extracting protein from 15 5x10<sup>6</sup> T cells isolated from the thymus or spleen in Lammelli buffer (53). These protein samples were then heated to 95C, loaded onto a 10% polyacrylamide SDS-containing gel and run at 100v for 2h or until the dye front reached the bottom. The protein was transferred 20 electrophoretically to Immobilon Membranes (Millipore, Bedford, MA). Membranes were blocked for 1h at room temperature using tris buffered saline (TBS) with 5% dried milk and 0.01% tween 20. Application of the primary antibody for either hCaMKIV or calspermin was 25 then carried out for 2h at room temperature in TBS followed by 6 washes in TBS containing 0.01% tween 20 (TBSt) lasting 10 min each. 1x10<sup>6</sup> cpm/ml of [<sup>125</sup>I] protein-A was then applied for 1h in TBS containing 5% dried milk and 0.01% tween 20 followed by 6 washes in 30 TBSt for 10 min each. Blots were exposed on Kodak XAR film overnight for visualization. CREB and phosphoCREB

detection was accomplished by stimulating 5x10<sup>6</sup> cells with PMA (10 ng/ml) and ionomycin (0.5 $\mu$ g/ml) or anti- $\alpha$ -CD3 monoclonal antibody (16 $\mu$ g/ml) that had been immobilized to 24 well tissue culture plates at 37C in 5 95% O<sub>2</sub>/5% CO<sub>2</sub>. At various time points T cells were harvested and lysed in Lammeli buffer as described previously. Proteins were then fractionated by SDS-PAGE on a 10% gel and subjected to western blotting as described above using commercial antibodies from Upstate 10 Biotechnology Incorporated (Lake Placid, NY). ECL chemiluminescent detection (Amersham, Arlington Heights, IL.) was substituted for [125I] protein-A following the established manufacturers protocols. Blots were quantified by scanning densitometry on a 445 SI 15 phosphorimager equipped with a personal densitometer SI (Molecular Dynamics, Sunnyvale, CA). Digital analysis of the scans was completed using the program Image Quant v1.1 (Molecular Dynamics, Sunnyvale, CA).

To measure production of IL-2, 2x10<sup>5</sup> T cells were 20 placed into a flat bottom 96 well plate (Corning-Costar, Cambridge, MA) containing the RPMI-1640 media previously described. Well volumes were then adjusted to 250ul and the cells were stimulated at 37C in a humidified chamber with PMA (10 ng/ml) and ionomycin (0.5 $\mu$ g/ml) or 16 $\mu$ g/ml 25 of the anti- $\alpha$ -CD3 monoclonal antibody that had been immobilized onto the plate. After 48h the media was removed for analysis. ELISA measurements were done following the standard protocol distributed with the ELISA components (Pharmingen, San Diego, CA) for 30 measurement of IL-2. Plates were then quantified on a

Multiskan MS 96 well plate reader (Lab Systems, Needham Heights, MA)

Statistical Analysis:

5 Initial statistics were performed by a two-way analysis of variance utilizing a 95% confidence level. Post-Hoc analysis was completed using either the Student-Newman-Keuls test when analyzing data from all three types of mice or a two tailed Students t test for data derived  
10 from just two types of mice. All data are presented as the mean  $\pm$  standard deviation. A level of significance was accepted when  $P<0.05$

EXAMPLE I

15 Generation of transgenic mice. Figure 1A presents diagrammatic representations of the constructs used to produce transgenic mice. The targeting construct is regulated by the proximal promoter of the murine lck gene and includes sequences of the human growth hormone gene at the 3' end to insure recognition by the splicing machinery. This construct has been shown to direct high level expression of cDNAs cloned between the lck and hGH sequences exclusively in thymic T cells (25, 27). The  
20 diagram on the left illustrates the CaMKIV construct. A single mutation was introduced at Lys-75 (Lys to Glu) of the human CaMKIV cDNA that renders the protein  
25 catalytically inactive (23, 28). On the right side of Figure 1A is shown the construct made to produce rat  
30 calspermin. Calspermin is a testis specific calmodulin binding protein that is composed of the C-terminal 169

amino acids of CaMKIV (29) and is generated from the CaMKIV gene by utilization of a promoter found in the penultimate intron of the gene (30, 31). Since the kinase inactive CaMKIV retains its high affinity for 5 Ca<sup>2+</sup>/calmodulin, the calspermin-containing mice were intended to distinguish between any possible dominant/negative effects of the mutant hCaMKIV and effects that might be produced as a consequence of nonspecific Ca<sup>2+</sup>/calmodulin sequestration. Both CaMKIV 10 and calspermin are localized predominantly in the nucleus.

Genotypic characterization of the transgenic mice is shown by the Southern blots shown in Figure 1B. The transgenic constructs were generated so that the entire 15 transgene could be removed by digestion with Spe1. This would produce a 6.9 kb fragment from integration of the hCaMKIV transgene and a 5.8 kb fragment from integration of the calspermin transgene. The left panel shows the results of a Southern analysis from DNA extracted from 20 two transgenic and two control mice. It is readily apparent that only the transgenic mice contain a single fragment of the predicted size that hybridizes to the CaMKIV probe. The right panel shows the equivalent experiment carried out on DNA isolated from one 25 transgenic mouse and three control littermates. Again the single predicted fragment is present only in the DNA from the transgenic mouse.

Western blot analysis was used to confirm that the appropriate protein was produced from the respective 30 transgenes. The left panel of Figure 1C shows the results obtained when an antibody is used that

recognizes both the endogenous mouse and transgenically produced human CaMKIV. Thymocyte protein from two transgenic mice flank a similar preparation from a control mouse. It can be seen that the expression level 5 of the transgenic protein is higher in the right lane (Line I) than in the left lane (Line K). Densitometry was used to quantify the difference in protein expression. The amount of immunoreactive CaMKIV in the Line I lane is 4x greater than in the control lane. 10 Thus the thymocytes from Line I produce about a 3x molar excess of the mutant hCaMKIV relative to endogenous levels of the authentic enzyme. Animals from Line I were used in all subsequent experiments. An antibody to a synthetic peptide containing the C-terminal 17 amino 15 acids of hCaMKIV was also prepared that is specific for the human form of the protein. As can be seen in the far left panel of Figure 1C only T cells resident in the thymus of the transgenic mouse contained the human protein. The fact that no transgenic product is present 20 in thymocytes isolated from the spleen of the identical transgenic mice, is consistent with the fact that the proximal lck promoter is not active once T cells are exported from the thymus (26). Additionally, the absence of the mutant hCaMKIV in splenic T cells implies 25 that this protein must be unstable in these cells.

The center panel of Figure 1C shows that thymocytes removed from the thymus of one line of transgenic mice produce considerable amounts of calspermin. As there are no amino acids present in calspermin that are not 30 also present in CaMKIV it has not been possible to produce an antibody that recognizes calspermin but not

CaMKIV. In addition the isoelectric pH of calspermin is 4.0 and consequently this protein does not transfer quantitatively under the conditions usually employed for Western blots (29). However, examination of the blots 5 suggests that the amount of calspermin present is in considerable excess to the amount of endogenous CaMKIV. Unquestionably the ratio is greater than 3:1. Therefore, these cells can be used to control for any nonspecific effects due to the presence of the mutant 10 hCaMKIV such as sequestration of Ca<sup>2+</sup>/calmodulin.

The presence of mutant hCaMKIV in thymocytes results in a developmental defect. The most obvious phenotypic consequence resulting from the expression of 15 the mutant hCaMKIV was a large reduction in the size and weight of the thymus. This gross phenotype was confirmed by analysis of the number of T cells resident in the thymus. As shown in Figure 2A (left panel) the total number of thymocytes present in the thymus of 20 transgenic mice is only about 16% of that found in the thymus of nontransgenic littermates or in transgenic mice that express calspermin (N=14, P<0.01). The deficit observed in the number of T cells in the thymus is completely normalized when the number of splenic 25 cells is quantified. As shown in the left panel of Figure 2B, no statistically significant difference exists between the number of splenocytes in control, transgenic hCaMKIV mutant (lck-CaMKIV Kin(-)) or transgenic calspermin (lck-calspermin) mice. This normalization in 30 the spleen is entirely consistent with the fact that splenocytes cease expression of the hCaMKIV transgene.

One explanation for the decreased cellularity of the thymus could be that maturation of T cells within that organ is defective. To examine this question, flow cytometry was used to quantify the number and 5 distribution of cells, based on the cell surface markers CD4 and CD8. The results of these experiments are shown as Figure 3. Cells were isolated from the thymus or spleen and an equivalent number of live cells ( $4 \times 10^6$ ) was aliquoted based on trypan blue exclusion followed 10 by counting in a hemocytometer. Thymic cells are shown in the top row of panels in Figure 3. Thymocytes that do not contain either cell surface marker are called double-negative and are distributed in the lower left quadrant. Double positive cells (CD4/CD8) are found in 15 the upper right quadrant. CD4 positive cells are sorted in the upper left and CD8 positive in the lower right. The numbers in the quadrants represent the percent of the total cells that are scored in that quadrant. It is readily seen that there are no differences in the 20 distribution of T cells in the thymus of hCaMKIV mutant, calspermin or control mice. The lower row of panels shows the same experiment carried out on splenocytes from the identical mice. Only single positive CD4 or CD8 T cells exit the thymus to become stored in the 25 spleen. So, the quadrants to compare are the upper left (CD4) and lower right (CD8). Again no differences are apparent between any of the three animals. The largest number of cells present in the spleen are B lymphocytes and are scored in the lower left quadrant as they do not 30 express either the CD4 or CD8 surface antigens.

The data in Figure 3 suggest that development and maturation of T cells are not impaired in the mice expressing the mutant hCaMKIV. However, the ability to generate or maintain normal numbers of T cells during 5 development has been compromised. This decreased cellularity could result from either a significant defect in proliferation and/or an increased rate of cell death of thymic T cells. The first attempt to address this question was to employ a cell survival assay. 10 These results for thymic and splenic T cells are presented in the upper and lower right hand histograms of Figure 2, respectively. Primary culture conditions were used such that 50-60% of T cells from the control mice will survive a 30 hr. incubation. Whereas there is 15 no difference between normal thymic T cells and those expressing calspermin, cells containing the mutant hCaMKIV undergo a much greater rate of spontaneous apoptosis ( $N=34, P<0.01$ ). This defect is not apparent in cells isolated from the spleen, a result entirely 20 consistent with the observation that splenic T cells no longer express the transgene product. Thus at least one explanation for the decreased cellularity found in the thymus of the mice expressing the mutant form of CaMKIV could be that they die more rapidly, at least in cell 25 culture.

The presence of mutant hCaMKIV results in a T cell signaling defect. It has been shown that CaMKIV is activated within seconds following engagement of the T 30 cell receptor (17, 23, 32). Similarly, CREB is rapidly phosphorylated in response to a T cell activating signal

(15). Since CaMKIV phosphorylates CREB on Ser-133 (19, 20, 21), it was determined whether the T cells that expressed the mutant hCaMKIV might have an altered ability to phosphorylate CREB. The results of a 5 representative experiment are shown in Figure 4. As can be seen in the top left hand panel, addition of phorbol ester (PMA) and ionomycin (shown as P/I as in the figure) to thymic T cells from normal mice resulted in robust phosphorylation of CREB by one min. This signal 10 was maintained for at least 30 min. The Ser-133 phosphorylated form of CREB was selectively examined by the use of an antibody that only recognizes this phosphorylated epitope (33). Similar results were obtained when the cells were stimulated by addition of 15 the anti-CD3 antibody which produces the signal through engagement of the T cell receptor (a-CD3). The location of the phosphoCREB signal is depicted by an arrow. Note that a faster migrating protein also shows an increased phosphorylation in response to addition of P/I 20 or a-CD3. This protein has been provisionally identified as ATF1, another member of the CREB family of transcription factors because: a) the phosphorylated form of ATF1 is known to be recognized by the phosphoCREB antibody; b) it is the same size as ATF1 25 based on migration in an SDS gel; and c) ATF1 has also been shown to be present in T cells (15) and can be phosphorylated and activated by CaMKIV (34). The third autoradiogram on the right shows that there was no change in the total amount of CREB during the 30 min. of 30 the experiment by use of an antibody that recognizes both phosphorylated and unphosphorylated CREB ( $\alpha$ -CREB).

The right panels at the top of Figure 4 show the results obtained when T cells containing the mutant hCaMKIV were stimulated with P/I or  $\alpha$ -CD3. Very little phosphorylation of CREB was observed at any of the 3 time points in response to either stimulus. In the case of ATF1, it was not possible to demonstrate any increase in phosphorylation. The third autoradiogram shows that equivalent amounts of CREB were present at all time points. Comparison of the  $\alpha$ -CREB blots between the 10 normal and mutant hCaMKIV-containing cells shows that the protein is also present in similar amounts in T cells from both types of mice. The abrogation of CREB phosphorylation characteristic of thymocytes from mice expressing the mutant hCaMKIV was not observed in T 15 cells containing the calspermin protein. Shown in the middle panels of Figure 4 is CREB phosphorylation in splenic T cells in response to  $\alpha$ -CD3. Cells from both control and mutant hCaMKIV mice show similar increases in phosphorylation of CREB. The recovery of a normal 20 response in cells from the transgenic mice is again consistent with the fact that splenocytes no longer contain the mutant hCaMKIV protein. Finally, the bottom panels of Figure 4 show that the presence of calspermin 25 does not affect phosphorylation of CREB in response to P/I stimulation. It was concluded that the presence of the mutant enzyme must result in a dominant/negative effect on CREB phosphorylation whether the stimulus is physiologically relevant like  $\alpha$ -CD3 or more general as elicited by the combination of PMA and ionomycin.

30 CREB phosphorylation in thymic T cells is required for the activation of the immediate early genes *cfos*,

Fra-2 and Fos B (15). Therefore, the appearance of Fos-B mRNA in response to T cell stimulation was examined. As shown in Figure 5, stimulation of control T cells with  $\alpha$ -CD3 resulted in transient expression of Fos B mRNA. The time course of this response and the presence of the two mRNA species are identical to the results reported by Barton et al., (15). However, cells that express the mutant hCaMKIV fail to demonstrate an increase in Fos B mRNA in response to stimulation. The 10 northern blot was stripped and reprobed with a GAPDH cDNA. The bottom autoradiogram in Figure 5 shows that each lane did contain equivalent amounts of RNA. Thus the presence of the mutant hCaMKIV correlates with the inability to phosphorylate CREB and to produce Fos B 15 mRNA.

The CREB/AP 1 pathway has been shown to be critical for the production of IL-2 (15). Figure 6A shows that whereas a 48 hr. stimulation of T cells from control or calspermin transgenic mice produce equivalent amounts of 20 IL-2 in response to  $\alpha$ -CD3, very little of the cytokine is made by the cells that contain the mutant hCaMKIV. As one consequence resulting from T cell activation is an increase in the number of cells that express the IL-2 receptor (15, 35, 36), we also examined the appearance 25 of the a chain of the IL-2 receptor (CD25) 30 hr after cells were challenged with a mitogenic stimulus. The results, shown in Figure 6B, show a similar response of control and calspermin-containing cells but a considerable reduction in cells that express mutant 30 hCaMKIV. Qualitative analysis of the data suggest that the former two cell populations show about a 10-fold

increase in the proportion that contain CD25, whereas less than a 3-fold increase is seen in the cells containing mutant hCaMKIV.

The data presented in Figures 4-6 are representative experiments. Each experiment was repeated several times with T cells from different transgenic mice. Because of the small numbers of such cells, it was not possible to conduct each experiment on cells from the same animals. Therefore, considerable variation was observed from animal to animal. This is due to the fact that the T cells contained differing amounts of the mutant hCaMKIV. A consistent observation was that the magnitude of the inhibitory responses was directly proportional to the amount of CaMKIV. In addition, when it was possible to carry out two assays on cells from the same animal, the proportional differences were identical when comparing CREB phosphorylation to IL-2 production or to the decrease in the number of cells expressing CD25. For example if CREB phosphorylation was decreased by 95%, similar percent decreases were found in either of the other two parameters. Based on these observations, it is believed that the 4 events, CREB phosphorylation, induction of Fos B mRNA, IL-2 production and the expression of CD25 are related to each other and are coincidentally down-regulated by the presence of the mutant hCaMKIV.

EXAMPLE II

Expression of CaMKIV kin(-) in T cells  
of Transgenic Mice

5

To examine the role of CaMKIV in T cells, transgenic mice were generated in which expression of a kinase minus version of CaMKIV was targeted to thymocytes. Since CaMKIV is not detectable at day 15 of embryonic development (E15), a T cell specific promoter was used that is expressed prior to this developmental time. The choice was the proximal promoter for the proto-oncogene lck. This promoter has been shown to target T cell specific expression in transgenic mice (Chaffin et al, EMBO J. 9:3821 (1990)) and, whereas it is not known precisely when the promoter becomes active during development of the thymus, it is expressed by E15 (Reynolds et al, Mol. Cell. Biol. 10:4266 (1990)). Thus, the utilization of the lck promoter maximizes any consequences of expressing the kinase minus CaMKIV since the transgene would be expressed prior to the endogenous gene during thymic development. In addition, the proximal lck promoter is active at all stages of T cell development in the thymus but is silenced when cells are released into the circulation and is also inactive in thymocytes that are present in the spleen (Chaffin et al, EMBO J. 9:3821 (1990)).

A lck promoter construct first characterized by Chaffin et al (EMBO J. 9:3821 (1990)) was used to target expression of CaMKIV or a kinase minus mutant form of CaMKIV to thymocytes in transgenic mice. A line of mice

has been developed that expresses about 2.5 fold more kin(-) protein than endogenous CaMKIV.

Remarkable effects were observed in the analysis of 25 day old animals. First, the thymus size was 5 decreased by 75% relative to mice that overexpressed the wt CaMKIV. This decrease in size was due to a similar % reduction in the total number of cells resident in the thymus. Second, the remaining T cell population contained a much greater proportion than normal of dead 10 cells. Cell sorting analysis confirmed that an increased number of apoptotic cells was present. However, in the live cell population, no gross differences in CD+, CD8+ or double positive cells were apparent. The number and cell surface marker 15 distribution of splenic T cells were normal, suggesting no defect in the export of cells from the thymus. These findings indicate that if the kin(-) protein is acting in a dominant/negative way, then the presence of active CaMKIV could either select against apoptosis or be 20 required for proliferation. It is unclear how kin(-) could behave as a dominant/negative but a similar effect of a kinase minus form of the MAP kinase, MEK-1, has been observed in mice using the same lck promoter based construct (Alberola-Illa et al, Nature 373:320 (1995)). 25 In the case of the MEK-1 kin(-), positive selection of T cells was inhibited as the very specific phenotype. In the present case, the results indicate that either saturation of a CaMKIV binding protein is occurring or inhibition of nuclear entry (and/or interactions) of the 30 kinase is being effected. The fact that the lck promoter is expressed earlier in development than is

CaMKIV creates ideal conditions to generate a dominant/negative effect.

The presence of the mice expressing the kin(-) CaMKIV in the thymocytes provided the opportunity to determine if the remaining live cells behaved differently in culture. Live cells were quantified and placed in culture. When thymic T cells were cultured in serum-containing medium they did not proliferate as they lack the co-stimulatory signals discussed above. Rather they undergo spontaneous apoptosis in a time-dependent manner. Incubation of T cells from a normal mouse for 30 hr. results in about 40% of the cells undergoing apoptosis. However, only 15% of the cells from the mice expressing the CaMKIV kin(-) protein survive. Thus the presence of kin(-) correlates with a 3-fold increase in the rate of spontaneous apoptosis. To examine whether CaMKIV plays a role in T cell activation, cells expressing kin(-) were stimulated with anti-CD3 antibody, phorbol ester, ionomycin or combinations of phorbol ester and ionomycin and IL-2 production evaluated. The cells secreted 6-fold less IL-2 in response to these treatments compared to T cells from normal mice of the same age. Similarly, phosphorylation of CREB was markedly attenuated when cells were challenged with agents that increased intracellular  $Ca^{2+}$  or activate PKC. These experiments indicate that CaMKIV is a CREB kinase in T cells and that this phosphorylation event provides a signal necessary for an early event in the cascade of reactions that result in T cell activation.

EXAMPLE III

Disruption of the CaMKIV Gene  
in Mice by Homologous Recombination

5

Mice in which the CaMKIV gene is silenced by homologous recombination have been produced. The DNA construct used to delete a portion of the gene in embryonic stem cells is shown in Fig. 7. The construct was prepared in collaboration with G. Stanley McKnight (University of Washington, Seattle). The construct was electroporated into embryonic stem cells at the NC Transgenic facility at UNC under the direction of Dr. Beverly Koller. The microinjection of the cells into blastocysts and the creation of the original founder mice was also done in that facility.

The primary phenotype of the first group of mice that are nullizygous for the CaMKIV locus, that is relevant to the invention, is that the mice lose all their hair within the first two weeks of life and appear very similar to nude mice. In the nude mouse, hair loss is directly related to the absence of T cells. These experiments confirm that CaMKIV is absolutely required for proper immune function and indicated that this enzyme is an excellent target for cell specific immunosuppressant drugs.

\* \* \*

All documents cited above and below are hereby  
30 incorporated in their entirety by reference.

One skilled in the art will appreciate from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention.

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WHAT IS CLAIMED IS:

1. A method of screening a test compound for immunosuppressive activity comprising:
  - i) contacting CaMKIV and a substrate therefor in the presence of said test compound, under conditions such that CaMKIV-dependent phosphorylation of said substrate can be effected, and
  - ii) determining the level of phosphorylation of said substrate resulting from step (i) and comparing said level with a level of phosphorylation of said substrate obtained in the absence of said test compound, wherein a reduction in the level of phosphorylation of said substrate in the presence of said test compound indicates that said compound has immunosuppressive activity.
2. The method according to claim 1 wherein said substrate is a peptide substrate.
3. The method according to claim 1 wherein said CaMKIV and said substrate are present in a cell free system.
4. The method according to claim 1 wherein said CaMKIV and said substrate are present in a cell.
5. The method according to claim 1 wherein, in step (i), said CaMKIV and said substrate therefor are contacted with said test compound in the presence of CaMKIV kinase, calmodulin and calcium under conditions

such that CaMKIV kinase dependent phosphorylation of CaMKIV can be effected.

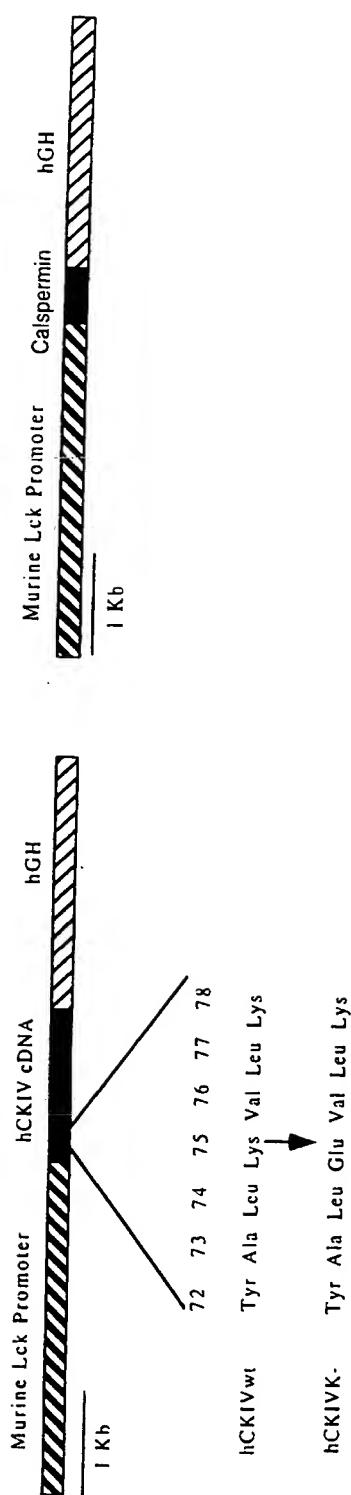
6. The method according to claim 5 wherein, in step (ii), the level of phosphorylation of CaMKIV is determined and compared with a level of phosphorylation of CaMKIV obtained in the absence of said test compound, wherein a reduction in the level of phosphorylation of said CaMKIV in the presence of said test compound indicates that said compound has immunosuppressive activity.

7. A non-human transgenic animal that comprises cells nullizygous for a CaMKIV locus.

8. The animal according to claim 7 wherein said animal is a rodent.

9. A non-human transgenic animal in which a kinase minus form of CaMKIV is targeted to the thymocytes thereof.

10. The animal according to claim 9 wherein said animal is a rodent.



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Fig. 1A

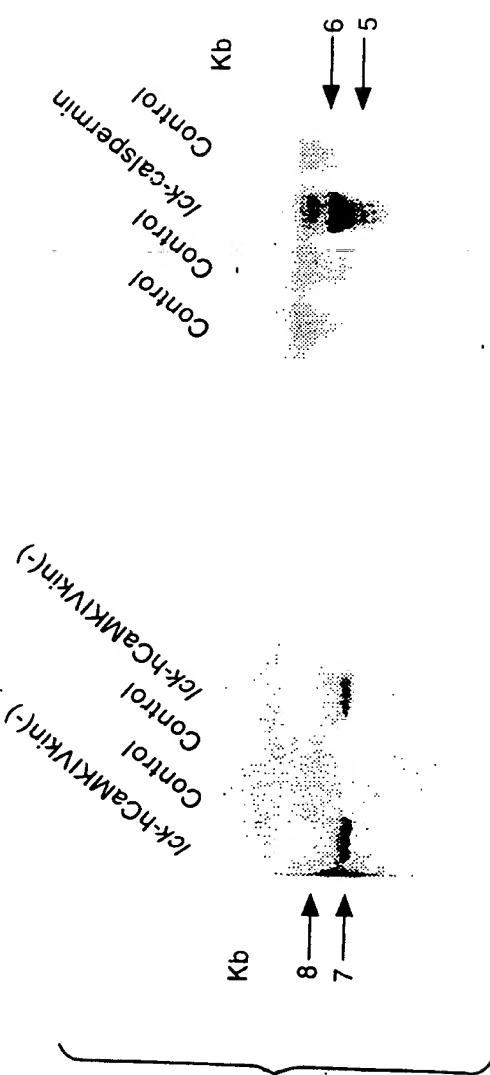
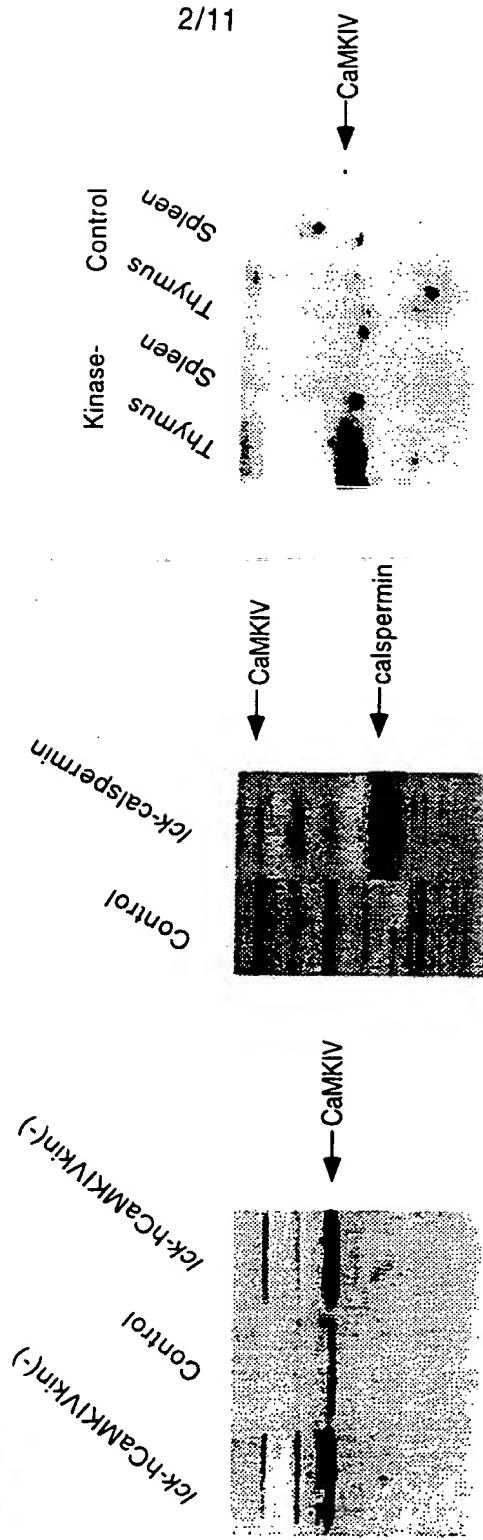


Fig. 1B

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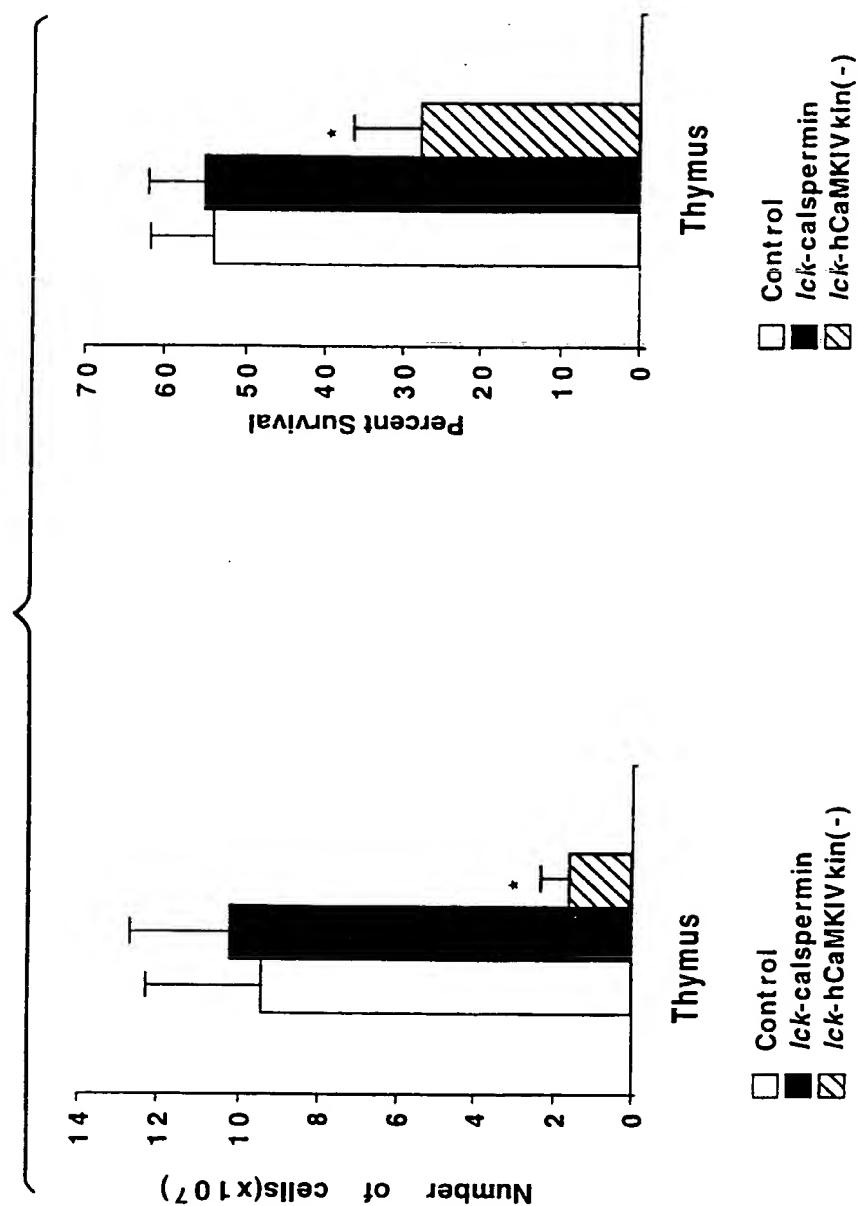
Fig. 1C



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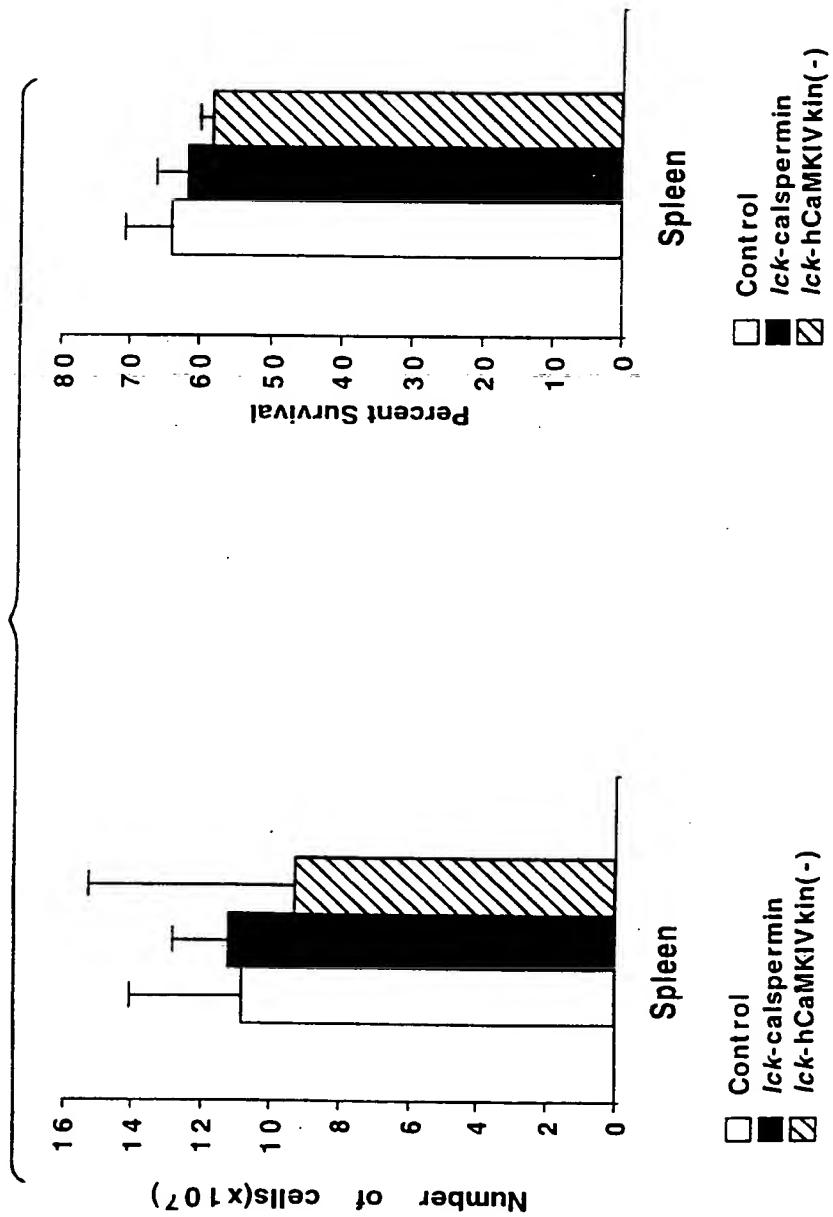
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Fig. 2A



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Fig. 2B



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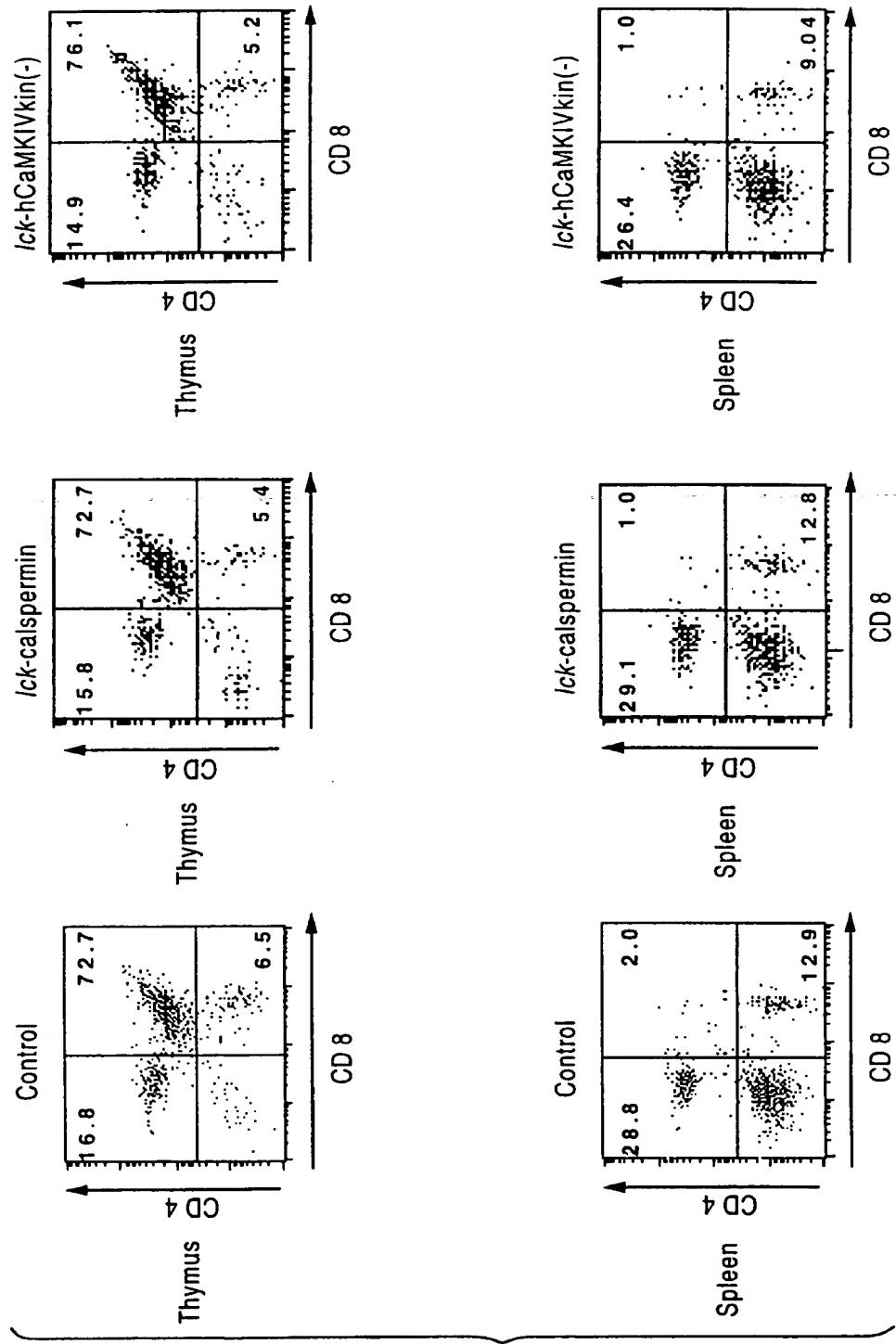
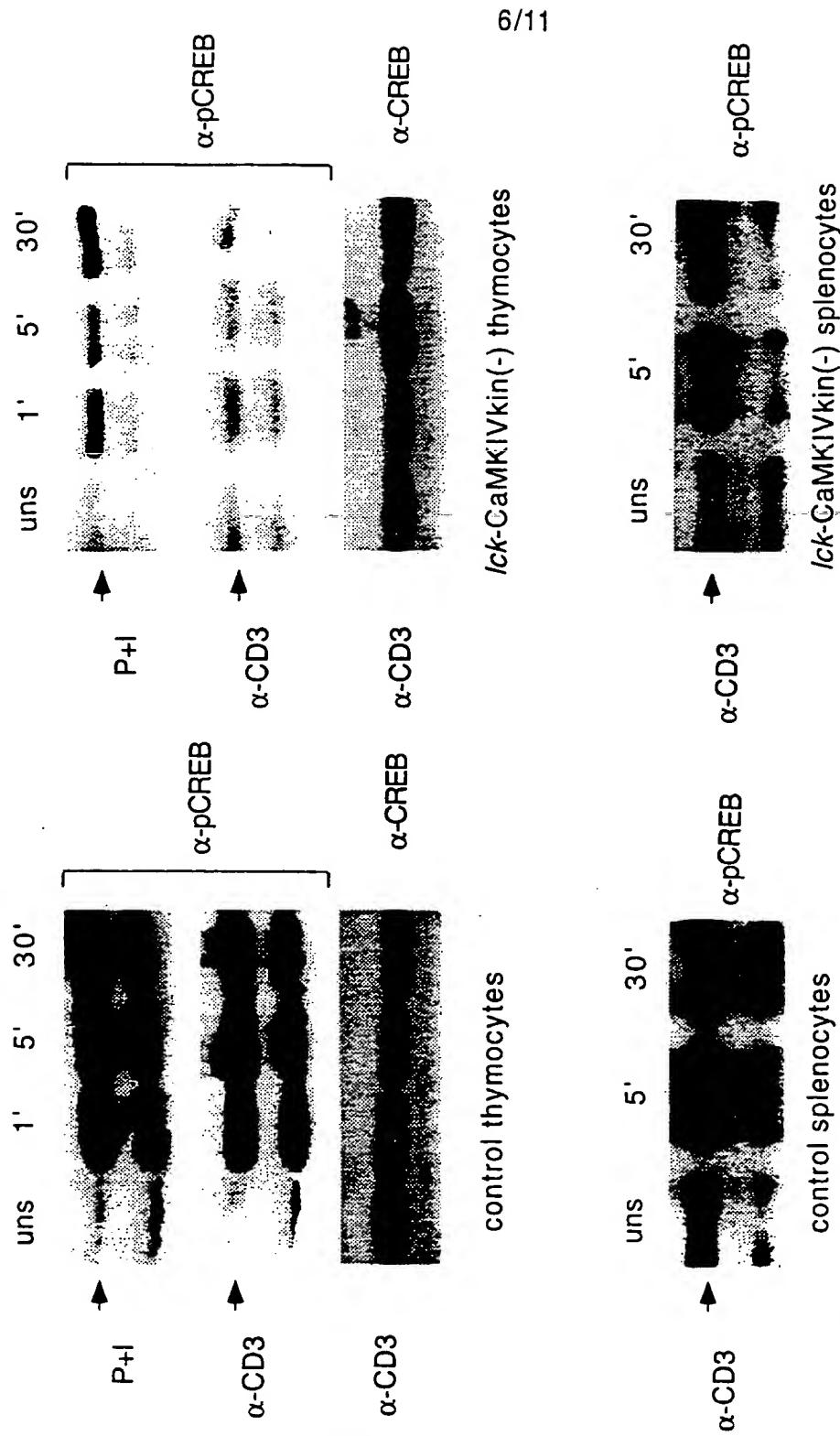


Fig. 3

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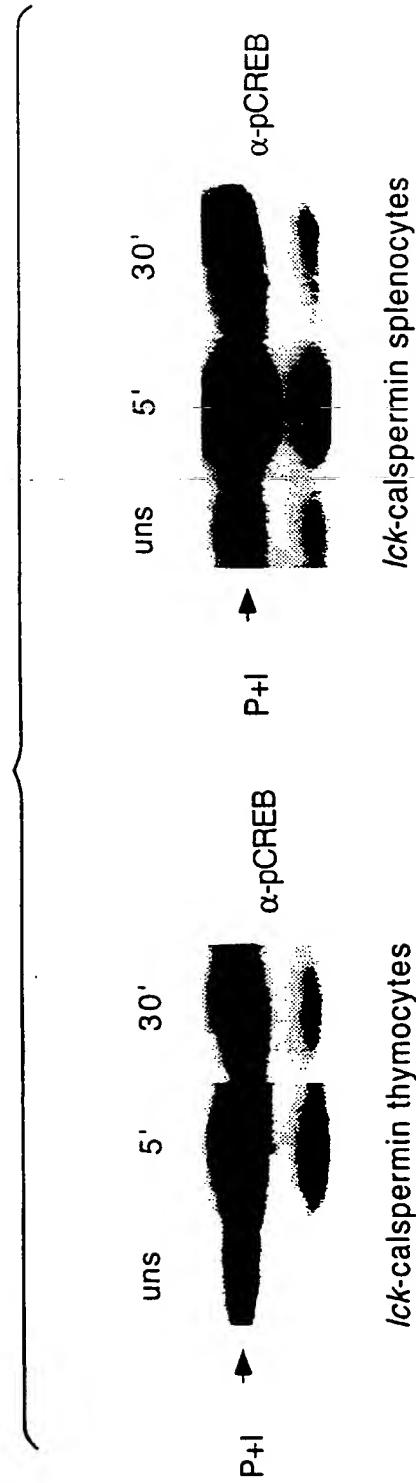


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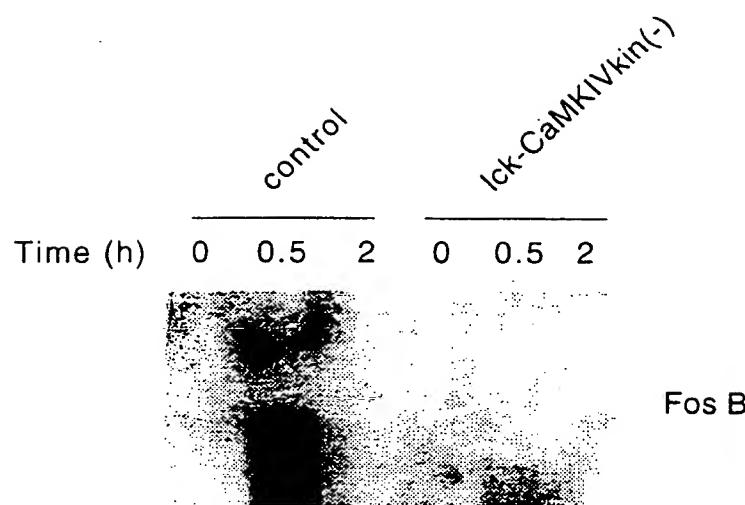
Fig. 4A

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Fig. 4B

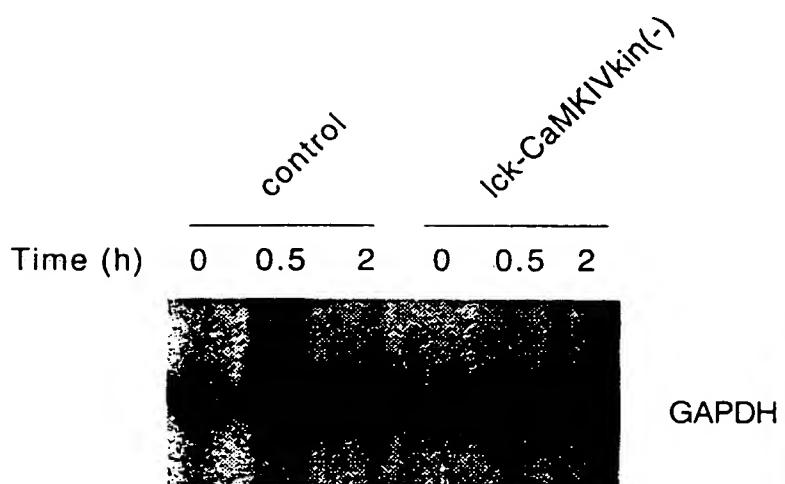


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Fos B

Fig. 5



GAPDH

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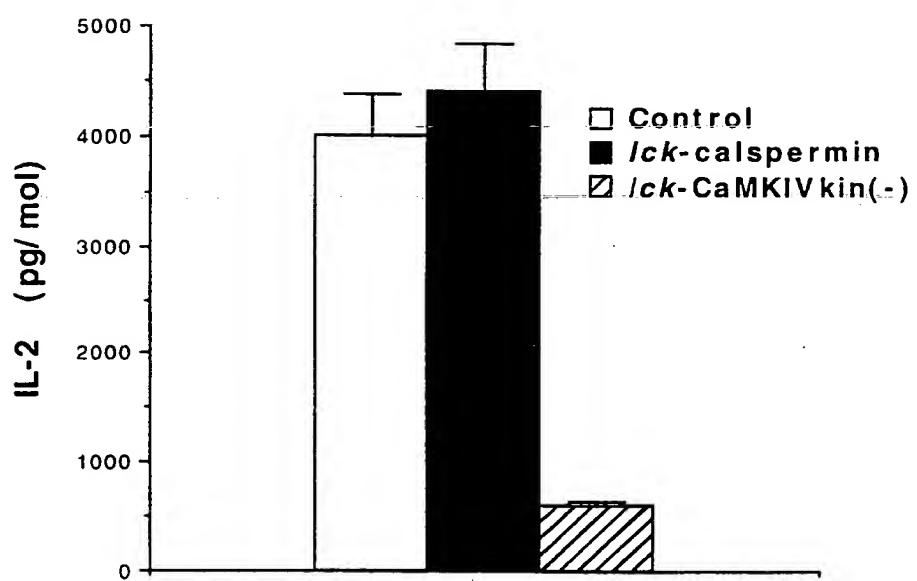


Fig. 6A

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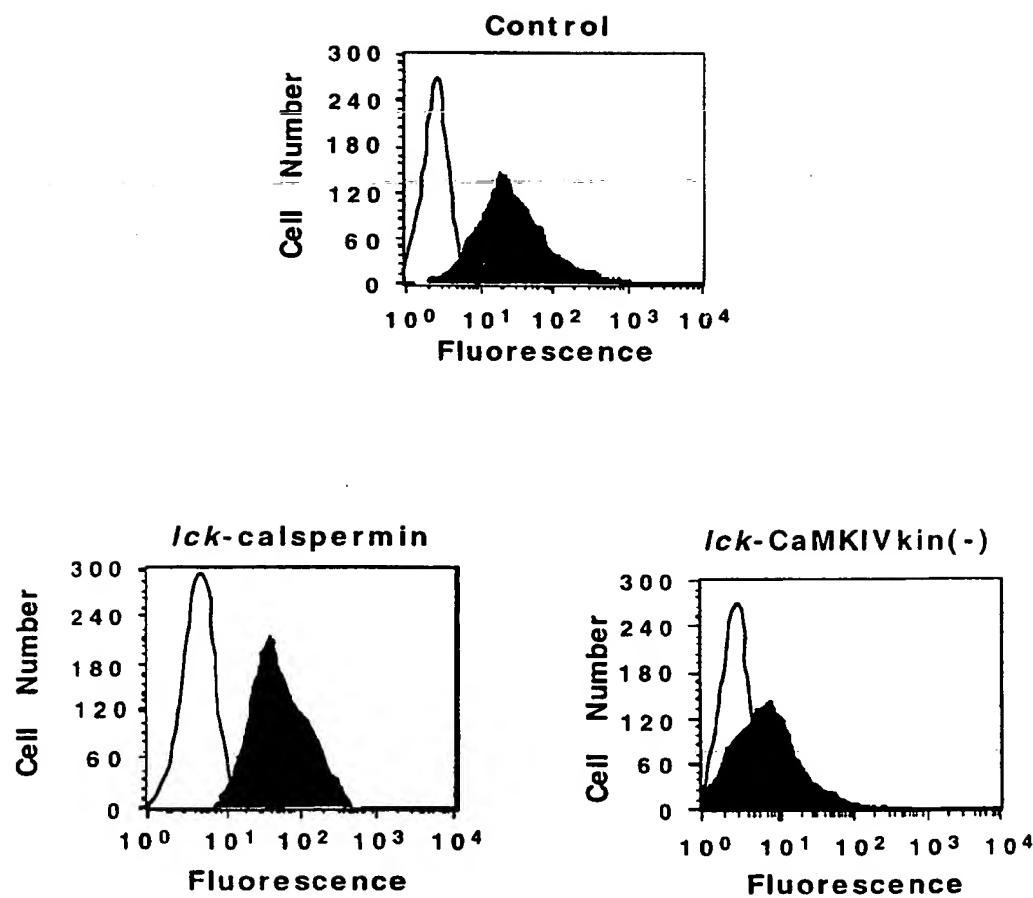
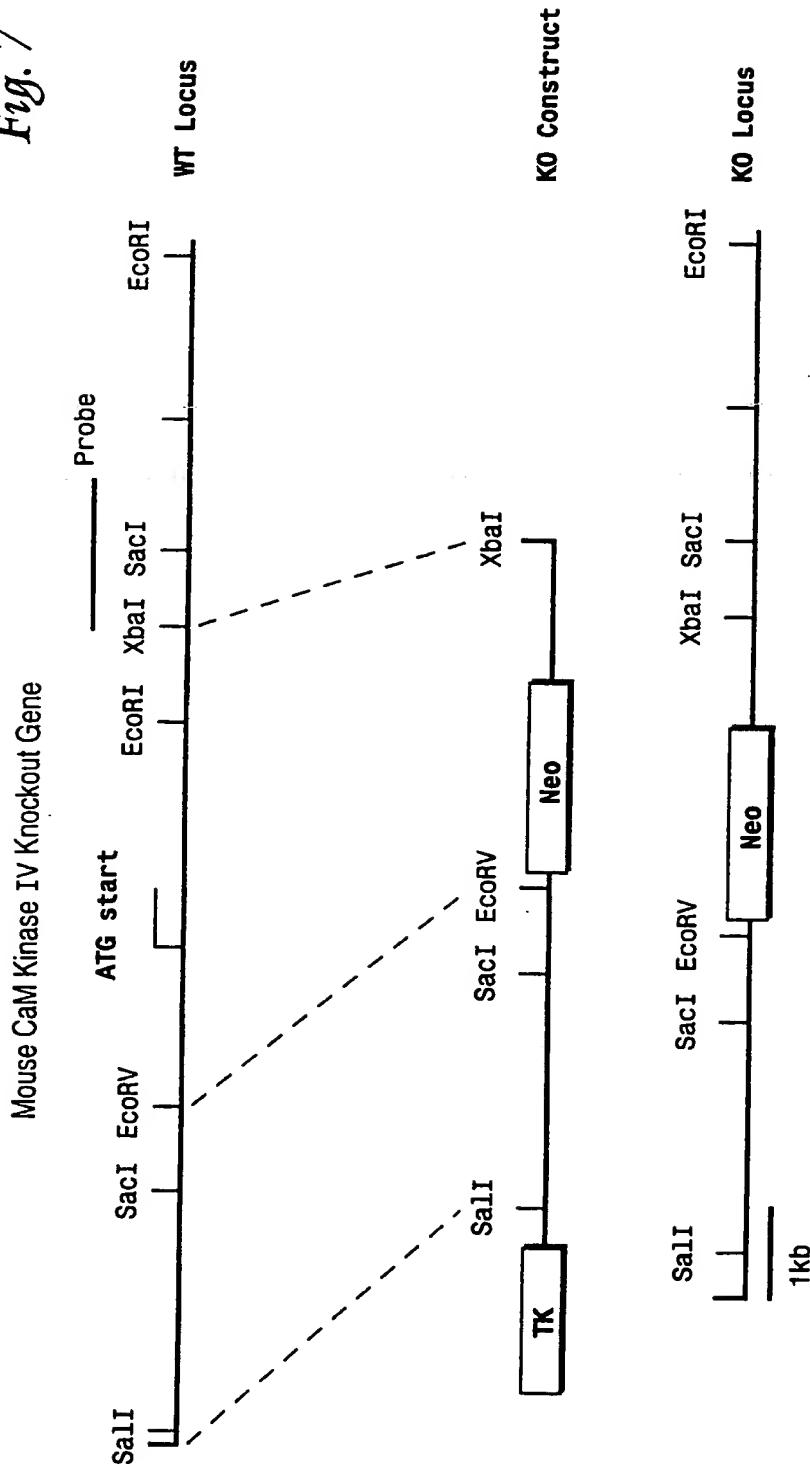


Fig. 6B

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Fig. 7



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/04205

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 5/00, 15/00, 15/09  
 US CL :800/2; 435/172.3, 69.1, 320.1, 325. 4  
 According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/2; 435/172.3, 69.1, 320.1, 325. 4

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category <sup>a</sup>	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CHATILA et al. A Unique Phosphorylation-dependent Mechanism for the Activation of Ca <sup>2+</sup> /Calmodulin-dependent Protein Kinase Type IV/GR. The Journal of Biological Chemistry. 30 August 1996, Vol. 271, No. 35, pages 21542-21548, see entire document.	1-10
Y	TOKUMITSU et al. Activation Mechanisms for Ca <sup>2+</sup> /Calmodulin-dependent Protein Kinase IV. The Journal of Biological Chemistry. 18 November 1994, Vol. 269, No. 46, pages 28640-28647, see entire document.	1-10
Y	SIKELA et al. Chromosomal Localization of the Human Gene for Brain Ca <sup>2+</sup> /Calmodulin-Dependent Protein Kinase Type IV. Genomics. 1989, Vol. 4, pages 21-27, see entire document.	7-10

Further documents are listed in the continuation of Box C.  See patent family annex.

* Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*B* earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reasons (as specified)	"A"	document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means		
*P* document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
20 MAY 1998	08.07.98
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JILL D. SCHMUCK Telephone No. (703) 308-0166

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/04205

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	OKUNO et al. Evidence for the Existence of $\text{Ca}^{2+}$ /Calmodulin-Dependent Protein Kinase IV Isoforms in Rat Brain. <i>Journal of Biochemistry</i> . 1996, Vol. 119, pages 1176-1181, see entire document.	1-10
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Y	ENSLEN et al. Characterization of $\text{Ca}^{2+}$ /Calmodulin-dependent Protein Kinase IV: Role in Transcriptional Regulation. <i>The Journal of Biological Chemistry</i> . 03 June 1994, Vol. 269, No. 22, pages 15520-15527, see entire document.	1-10
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Y	BUTLER et al. Limbic Epilepsy in Transgenic Mice Carrying a $\text{Ca}^{2+}$ /Calmodulin-dependent Kinase II $\alpha$ -Subunit Mutation. <i>Proceedings of the National Academy of Sciences, USA</i> . July 1995, Vol. 92, pages 6852-6855, see entire document.	7-10

INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/04205

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

MEDLINE, BIOSIS, CAPLUS, EMBASE, WPIDS, APS

search terms: CAMKIV, Ca2+ calmodulin dependent protein kinase IV, CAM-kinase IV, transgene, homologous recombination, knockout, disrupt, immunosuppress, reduce phosphorylation